Exhibit 4

TrpC1 Is a Membrane-Spanning Subunit of Store-Operated Ca^{2+} Channels in Native Vascular Smooth Muscle Cells

Shang-Zhong Xu, David J. Beech

Abstract—Mammalian counterparts of the *Drosophila trp* gene have been suggested to encode store-operated Ca^{2+} channels. These specialized channels are widely distributed and may have a general function to reload Ca^{2+} into sarcoplasmic reticulum as well as specific functions, including the control of cell proliferation and muscle contraction. Heterologous expression of mammalian *trp* genes enhances or generates Ca^{2+} channel activity, but the crucial question of whether any of the genes encode native subunits of store-operated channels remains unanswered. We have investigated if TrpC1 protein (encoded by *trp1* gene) is a store-operated channel in freshly isolated smooth muscle cells of resistance arterioles, arteries, and veins from human, mouse, or rabbit. Messenger RNA encoding TrpC1 was broadly expressed. TrpC1-specific antibody targeted to peptide predicted to contribute to the outer vestibule of TrpC1 channels revealed that TrpC1 is localized to the plasma membrane and has an extracellular domain. Peptide-specific binding of the antibody had a functional effect, selectively blocking store-operated Ca^{2+} channel activity. The antibody is a powerful new tool for the study of mammalian *trp1* gene product. The study shows that TrpC1 is a novel physiological Ca^{2+} channel subunit in arterial smooth muscle cells. **(*Circ Res.* 2001;88:84-87.)**

Key Words: calcium channel ■ blood vessel ■ artery ■ vascular smooth muscle

Store-operated channels (SOCs) are plasma-membrane Ca^{2+} channels that open when Ca^{2+} levels in sarcoplasmic or endoplasmic reticulum are depleted.[1,2] They are also referred to as capacitative Ca^{2+} entry channels, and highly Ca^{2+}-selective SOCs are called CRAC (Ca^{2+} release–activated Ca^{2+}) channels.[1-3] SOCs may serve an essential housekeeping function to refill sarcoplasmic reticulum after Ca^{2+} release.[2] SOCs may also be involved in muscle contraction,[1,4] control of proliferation in smooth muscle cells,[5] and CD95-mediated cell apoptosis.[6,7]

Knowledge of the molecular basis of SOCs is of fundamental importance for the understanding of Ca^{2+} signaling. One suggestion is that SOCs are products of mammalian *trp* genes (related to *Drosophila trp/trpl* genes).[8,9] Expressed *trp3* induces channel activity associated with store depletion, but it requires coactivation of receptors or diacylglycerol.[10] Trp4 is suggested to be an SOC[11] but is also described as a receptor-operated channel that cannot be activated by store depletion.[12] Expressed *trp1* may behave as an SOC,[13] but it is also reported to be a basally active channel independent of Ca^{2+} stores[14] and a diacylglycerol-activated channel.[15] These studies indicate that *trp* gene products are associated with SOCs, but evidence is lacking that they are membrane-spanning subunits or SOCs in native mammalian cells.[16,17] We sought to determine if TrpC1 (the mammalian *trp1* gene product) is a SOC in vascular smooth muscle cells.

Materials and Methods

Human left internal mammary arteries (LIMAs) and aortas were obtained with ethical approval from the Leeds Teaching Hospitals Local Research Ethics Committee. Vessels were placed in Hanks' solution containing (in mmol/L) NaCl 137, KCl 5.4, $CaCl_2$ 0.01, NaH_2PO_4 0.34, K_2HPO_4 0.44, D-glucose 8, and HEPES 5. Other vessels were from male Wistar rats or BalbC mice. Arteriole fragments were obtained from pial membrane.[18] Cells and vessels were stored at 4°C in Hanks' solution (<13 hours). mRNA was isolated with Dynabeads Oligo $(dT)_{25}$ (Dynal). Bead complexes were washed and transferred to 20 μL SuperScript RNase H^- Reverse Transcriptase (Gibco-BRL) at 42°C (30 minutes). Primers for *trp1* were TGGTATGAAGGGTTGGAAGAC (forward) and GGTATCATTGCTTTGCTGTTC (reverse). Primers for β-actin detection were TTGTAACCAACTGGGACGATATG (forward) and GATCTTGATCTTCATGGTGCTGG (reverse). Thermal cycling was 95°C (5 minutes), 40 cycles at 94°C (30 seconds), 53°C to 60°C (1 minute), 72°C (1 minute), and 72°C (7 minutes). Products were detected on 1.5% agarose gels and directly sequenced for rabbit pial membrane. T1E3 antibody was prepared in rabbit by Sigma-Genosys (UK) and targeted to TrpC1 sequence (Figure 2). Specificity was tested by ELISA, and preimmune serum had no activity. T1E3 antiserum was used at 1:500 dilution, and antigenic peptide was used at 10 μmol/L. For experiments in 100 mmol/L K^+, antiserum was cleaned on a HiTrap protein A column and used at 1:100 dilution with and without antigenic peptide at 20 μmol/L. For Western blotting, tissues were placed in 100 μmol/L phenylmethylsulphonylfluoride (Sigma) and lysed in SDS buffer containing 100 μmol/L dithiothreitol at 80°C to 100°C (15 minutes). Proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose, which was rinsed with PBS and incubated in PBS containing 10% milk for 1 hour (room temperature). Incubation in T1E3 was overnight at 4°C, followed by washes in PBS and incubation in horseradish peroxidase-secondary antibody (1:5000, BioRad) for 1 hour (room temperature). Membranes were washed with PBS, and labeling was detected by ECLplus (Amersham). For immunofluorescence, tissues and cells adhered to poly-L-lysine–coated slides were incubated in 1% BSA/PBS and transferred to T1E3 antibody for 12 hours and

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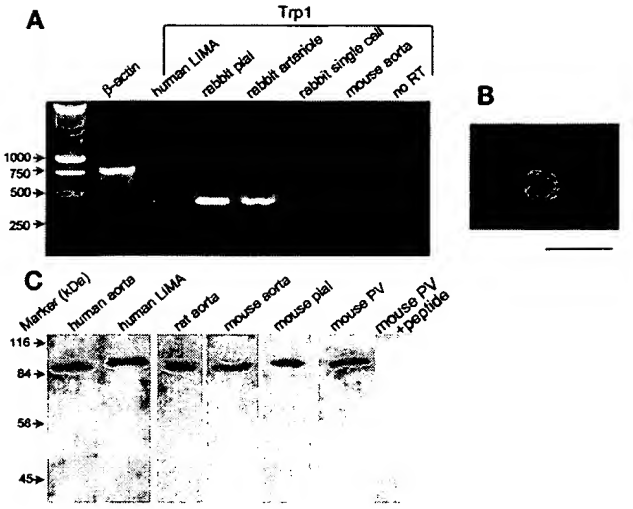

Figure 1. TrpC1 expressed in blood vessels. A, mRNA detected by RT-PCR in human LIMA, mouse aorta, and rabbit pial membrane (β-actin and *trp1*), arteriolar fragments, and single-cell rings. Predicted sizes of the *trp1* and β-actin PCR products were 423 and 763 bp. Products were not detected if reverse transcriptase was omitted, as shown for rabbit pial (no RT). B, Rabbit arteriolar smooth muscle ring stained with anti–α-SMA-Cy3. Scale bar=50 μm. C, Western blots with T1E3 antibody for aorta, LIMA, portal vein (PV), and pial membrane. Labeling was competed off by peptide in human vessels and mouse pial membrane (not shown) and PV (shown).

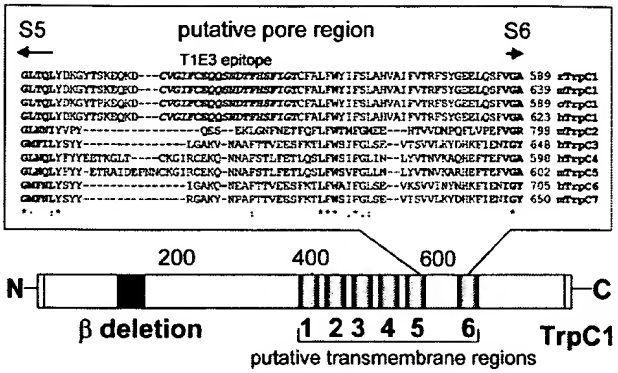

Figure 2. Targeting of T1E3 antibody to the outer vestibule of TrpC1 channels. Sequence alignment of putative pore regions of *trp* gene products between 5th and 6th membrane-spanning domains (S5 and S6). Accession numbers are AF061266 (rTrpC1), U73625 (mTrpC1), AF170493 (oTrpC1), U31110 (hTrpC1), AF111107 (mTrpC2), U47050 (hTrpC3), AF170456 (hTrpC4), AF029983 (mTrpC5), AF080394 (hTrpC6), and AF139923 (mTrpC7). r indicates rat; m, mouse; h, human; and o, rabbit.

secondary antibody (mouse anti-rabbit IgG-FITC, 1:160, Sigma) for 1 hour. Cells were identified with anti–smooth muscle α-actin antibody (anti–α-SMA-Cy3, 1:200, Sigma). Microscopy images were processed with Openlab software (Improvision). Permeabilized cells were fixed in 2% paraformaldehyde (30 minutes) and immersed in −20°C methanol (1 minute) and 1% BSA with 0.1% Triton X-100 for 1 hour. Ratiometric $[Ca^{2+}]_i$ or $[Ba^{2+}]_i$ measurements were as described[19] but using 340/380 nm excitation, and background fluorescence was subtracted. The superfusion solution contained (in mmol/L) NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10, and glucose 8; pH 7.4; flow rate was 5 mL/min. Ca^{2+} was replaced by 0.4 mmol/L EGTA for Ca^{2+}-free solution. All solutions included methoxyverapamil (10 μmol/L). For imaging experiments except those in 100 mmol/L K^+ solution, preincubation with 1:500 T1E3 was for 8 to 12 hours (4°C). When 100 mmol/L KCl replaced 100 mmol/L NaCl in the superfusion solution, arterioles were preincubated with 1:100 cleaned T1E3 for 2 hours (37°C). Antiserum and peptide were not in recording solutions. Recordings were made alternately from test and control cells. Signals were measured from 5 smooth muscle cells in each arteriole. Data are expressed as mean±SEM, and n is the number of cells. Comparisons were made using unpaired Student's *t* test.

Results

We probed for *trp1* mRNA expression in a range of blood vessels using reverse transcriptase–polymerase chain reaction (RT-PCR) and found that it was present (Figure 1A). It was also detected in single arteriolar smooth muscle cells harvested by a micro-hooking method (Figures 1A and 1B). TrpC1 protein was detected using a polyclonal antibody (T1E3) targeted to a mammalian TrpC1-specific peptide (Figure 2). The peptide was predicted to be extracellular on the basis of results of transmembrane detection algorithms (not shown) and studies of TrpC3 glycosylation[20] and *Xenopus laevis trp* expression.[21] Western blotting revealed that T1E3 is specific for protein of the mass predicted for TrpC1 (Figure 1C), which is 92 kDa for α-splice variant and 87 kDa for β-deletion (human TrpC1). Labeling by T1E3 was peptide-specific because it was absent after preadsorption to antigenic peptide (Figure 1C). Small variations in the size of labeled proteins may be explained by varying levels of α- and β-variants,[13] both of which were detected by RT-PCR (S.-Z.X. and D.J.B., unpublished data, April 2000).

Membrane-inserted TrpC1 protein was labeled with T1E3 as shown by immunofluorescence staining of permeabilized cells in arterioles (Figure 3A). Staining was most intense at the edge of smooth muscle cells in arterioles (Figure 3A) or in cells cultured from human LIMAs (Figure 3D), suggesting plasma membrane localization. Staining was specific because it was absent if T1E3 was preadsorbed to its antigenic peptide (Figures 3B and 3C). T1E3 antibody should also label unpermeabilized cells if the epitope is extracellular. Smooth muscle cells in enzymatically isolated rabbit pial arterioles were incubated with T1E3 before fixation with paraformaldehyde and without Triton-X permeabilization. Specific staining with T1E3 was detected and was most intense at the cell perimeter (Figure 3E). The absence of permeabilization was confirmed by lack of staining by anti–α-SMA-Cy3 (data not shown). Fluorescence was absent from rabbit arterioles incubated with secondary antibody but not T1E3 or T1E3+peptide (data not shown). Immunofluorescence studies were performed on rabbit as well as mouse because we could not satisfactorily isolate cells from mouse. Isolated rabbit arterioles are amenable to Ca^{2+} imaging, and we have evidence for SOCs.[19]

Sequence alignments of TrpCs with *Shaker* K^+ channel and related channels suggest that TrpCs are channel subunits. These alignments place the T1E3 epitope in the putative outer vestibule of the channel (Figure 2). Because antibodies targeted to this region of K^+ channels block K^+ currents,[22] we tested whether T1E3 inhibits Ca^{2+} entry. With block of voltage-gated Ca^{2+} channels and after store depletion caused by thapsigargin, reintroduction of extracellular Ca^{2+} caused a lanthanum-sensitive rise in $[Ca^{2+}]_i$ (Figure 4A) that was similar to that described previously.[19] The effect of reintro-

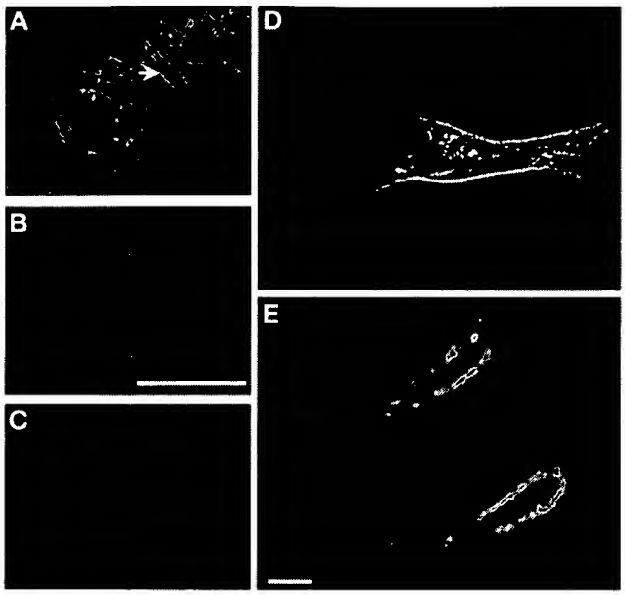

Figure 3. TrpC1 is expressed in native cells and spans the plasma membrane. A, Permeabilized mouse pial arteriole labeled with T1E3 antibody. The edge of one smooth muscle cell is indicated with an arrow. B, Another mouse arteriole stained with anti-α-SMA-Cy3. Scale bar=50 μm and applies to images in panels A through D. C, FITC fluorescence from the same arteriole as in panel B, which had also been incubated with T1E3 preadsorbed to antigenic peptide. D, Permeabilized cultured human LIMA smooth muscle cell double-labeled with T1E3 antibody (green) and anti-α-SMA-Cy3 (red). E, Live-cell staining of an enzymatically isolated rabbit arteriole stained with T1E3 antibody. Two smooth muscle cells are in the focal plane, and fluorescence is observed only at the perimeter of each cell. Scale bar=5 μm.

ducing Ca^{2+} was significantly larger after thapsigargin treatment (Figure 4B), suggesting a component of Ca^{2+} influx through SOCs. To test the effect of T1E3 on SOCs, arterioles were incubated with T1E3 at 4°C to allow binding of antibody but minimize de novo protein expression. In thapsigargin-treated (but not untreated) arterioles, the $[Ca^{2+}]_i$ signal on reapplication of Ca^{2+} was significantly smaller after incubation with T1E3 compared with incubation with T1E3 preadsorbed to antigenic peptide (Figure 4B). Ba^{2+} is permeant in Ca^{2+} channels but is weakly extruded or sequestered by cells. Thus, application of Ba^{2+} may permit a better measure of ion flux through SOCs. Ba^{2+} influx was measured after thapsigargin treatment and was significantly smaller after incubation in T1E3 without antigenic peptide (Figure 4C). The effect of T1E3 did not result from an effect on membrane potential, because T1E3 also inhibited Ba^{2+} influx when arterioles were studied in solution containing 100 mmol/L K^+, which strongly depolarizes and clamps the membrane potential (data not shown). In this condition, Ba^{2+}-induced $\Delta F_{340}/F_{380}$ was again significantly smaller in the T1E3 compared with the T1E3+peptide group (0.1728 ± 0.0054, n=50 versus 0.2153 ± 0.0112, n=40, $P<0.0005$). Antigenic peptide alone had no effect on Ba^{2+} flux: $\Delta F_{340}/F_{380}$ was 0.219 ± 0.007 in control and 0.211 ± 0.008 in peptide (n=75 for each, $P>0.05$).

Discussion

We show that TrpC1 is a novel type of Ca^{2+} channel in mammalian vascular smooth muscle and that a *trp* gene encodes a native store-operated channel.

That SERCA inhibition increased the intracellular Ca^{2+} signal on reintroduction of extracellular Ca^{2+} is suggestive of SOC activity,[19] although the effect could be explained by the superficial buffer barrier hypothesis[23] with constant background Ca^{2+} entry. We now show the effect of T1E3 on background Ca^{2+} signal alone and the signal plus that induced by SERCA inhibition. Importantly, T1E3 sensitivity depended on thapsigargin treatment. Thus, TrpC1 is not a background Ca^{2+} channel but a Ca^{2+} channel activated by store depletion.

Three observations demonstrate TrpC1 is a plasma-membrane protein spanning the membrane with an extracellular domain. T1E3 labeling is most intense at the cell perimeter. T1E3 labeled cells that were not permeabilized. Incubation of live cells with T1E3 inhibited Ca^{2+} entry. In the latter 2 cases, T1E3 must have bound an extracellular site. The Ca^{2+}/Ba^{2+} measurements additionally suggest that TrpC1 is a pore-forming subunit, because the T1E3 epitope is in the predicted outer vestibule of the channel. Although the blocking effect of T1E3 might seem relatively small, the effect was statistically significant in 3 independent data sets. Furthermore, a large block was not expected. First, only part of the Ca^{2+}/Ba^{2+} influx was store-operated. Second, a large antibody molecule is unlikely to be an efficient channel blocker. Third, we incubated with T1E3 for relatively short periods (8 to 12 hours at 4°C or 2 hours at 37°C) to minimize changes to native protein levels or cellular localization. Although T1E3

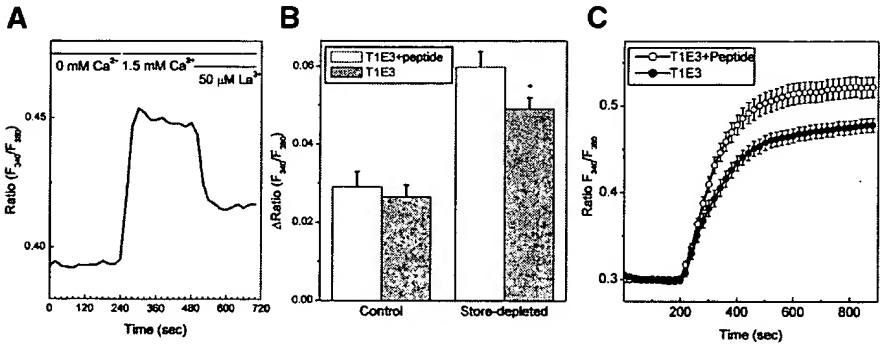

Figure 4. TrpC1 is a store-operated Ca^{2+} channel. A, La^{3+}-sensitive Ca^{2+} entry in a rabbit arteriolar cell after 1 hour in thapsigargin (1 μmol/L) (store-depleted). B, Stores were depleted, and the Ca^{2+} signal occurring on reintroduction of 1.5 mmol/L Ca^{2+} (as in panel A) was smaller with T1E3 compared with T1E3+peptide (n=95 each, $^*P<0.05$). Without store depletion, there was no effect of T1E3 (n=70 each). $N\omega$-nitro-L-arginine methyl ester (0.3 mmol/L) was included in panels A and B to inhibit basal nitric oxide production. C, As in panel A, stores were depleted. The signal occurred after addition of 10 mmol/L Ba^{2+} (200 seconds), and data are mean±SEM. (n=55 for each). The signal was smaller with T1E3 compared with T1E3+peptide ($^{**}P<0.001$ at 800 seconds).

was washed out before Ca^{2+} measurements, it remained bound, as demonstrated by immunostaining and Western blot.

There is evidence in addition to ours suggesting that TrpC1 is a SOC. It has been shown that human submandibular gland (HSG) cells transfected with HA-tagged *trp1* express a plasma-membrane localized protein.[24] Also, *trp1* transfection enhanced the Ca^{2+}-reentry signal in HSG cells treated with thapsigargin, and expression of *trp1* cDNA in antisense orientation inhibited basal Ca^{2+}-reentry signal in nontransfected HSG cells.[24] The *Xenopus* TrpC, which is similar in amino acid sequence to mammalian TrpC1, is localized to the plasma membrane of oocytes and HeLa cells.[21] Heterologous expression indicates TrpC1 is a channel subunit or that it can enhance activity of native channels, but it is unclear if the activity is that of a SOC.[13–15,24] TrpC1 seems to be a Ca^{2+}-permeable cation channel, but it is not highly Ca^{2+}-selective, and, thus, it is unlikely to be a CRAC channel. Intriguingly, SOCs in mouse aortic smooth muscle cells are nonselective cation channels like TrpC1 channels.[25]

There is evidence that *Drosophila* TRP and TRP/TRPL heteromultimers can form SOCs[26] and that the C-terminus of TRP provides thapsigargin sensitivity.[27] Intriguingly, TrpC1 is a smaller protein than *Drosophila* TRP or TRPL, with a shorter C-terminus. For this reason, it was predicted that TrpC1 is unlikely to be an SOC.[28] Our conclusions are at odds with this prediction and raise the question as to how TrpC1 couples to Ca^{2+} stores. We suggest, first, that TrpC1 is one pore-forming subunit in an SOC heteromultimer, another subunit having a longer C-terminus. Second, there is more than one mechanism by which SOCs can couple to Ca^{2+} stores, and the mechanism involving TrpC1 is different from that involving *Drosophila* TRP. There is evidence for TrpC heteromultimers[15] and multiple coupling mechanisms.[25,29,30]

We describe an antibody that is a powerful tool for studying TrpC1 effects and demonstrate that *trp1* gene encodes a novel channel subunit, contributing to store-operated Ca^{2+} channels in native arterial smooth muscle cells. TrpC1 is a potential target for novel drugs to alleviate hypertension or vasospasm or inhibit smooth muscle proliferation in arteriosclerosis and neointimal hyperplasia.

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